Award Number: W81XWH-12-1-0059

TITLE: Educating normal breast mucosa to prevent breast cancer

PRINCIPAL INVESTIGATOR: Keith L Knutson

CONTRACTING ORGANIZATION: Vaccine and Gene Therapy Institute
Port St Lucie, FL 34987-2352

REPORT DATE: May 2015

TYPE OF REPORT: Annual report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
May 2015

2. REPORT TYPE
Annual

3. DATES COVERED
24 April 2014 - 30 April 2015

4. TITLE AND SUBTITLE
Educating Normal Breast Mucosa to Prevent Breast Cancer

5a. CONTRACT NUMBER
W81XWH-12-1-0059

5b. GRANT NUMBER
W81XWH-12-1-0059

6. AUTHOR(S)
Keith L Knutson

E-Mail: Knutson.keith@mayo.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Vaccine and Gene Therapy Institute
9801 SW Discovery Way
Port St. Lucie, FL 34987

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Breast cancer develops from breast mucosa and breast mucosa has intact immune system to maintain epithelial integrity. In this study our goal was to study the immune subsets associated with breast mucosa and develop the strategies to populate mucosa with immune effectors in order to prevent breast cancer. Data obtained from our studies suggest that T cells constitute the majority of immune cells in breast mucosa and this includes conventional CD4 T cells, CD8+ αβ T cells and significant fraction of unconventional double positive (DP) CD4+CD8+ αβ T cells. We also observed that intramammary immunization induces antigen-specific immune responses in breast mucosa. Currently, studies are being done to characterize these double positive T cells to determine whether these are regulatory or cytotoxic in nature and their role in prevention of breast cancer. In addition to this, we are also investigating the ability of intramammary immunization in prevention of breast cancer and the feasibility of translating this approach into preventive breast cancer vaccine setting.

15. SUBJECT TERMS
Intraepithelial lymphocytes, Theilers murine encephalomyelitis virus, rat neu, intra mammary immunization, T cells

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction: The prevention of breast cancer is an active area of research in many countries and several different agents have been examined, notably estrogen antagonists. While these agents can be effective in high risk individuals, they only reduce the risk of specific subtypes of cancer and moreover these currently available drugs are associated with significant side effects and must be consumed daily and over years. Thus, an alternative approach to primary prevention of breast cancer is to train the mammary gland mucosal immune system to recognize and destroy potentially malignant cells based on overexpression of known tumor antigens. Rationale for this approach comes from the fact that breast cancer originates from a mucosal tissue which is endowed with large number of immune effectors and prior reports based on the effect of commensal bacteria on gut mucosa which suggests that it's feasible to educate gut mucosa in generating effective mucosal immunity. Our overall goal is to develop a preventative vaccination strategy to reduce the incidence and mortality from breast cancer based on training the mucosal immune system to detect potentially malignant cells early in the course of the disease. One important first step towards attaining this unique objective is to have a comprehensive knowledge about the breast mucosal immune system i.e. a profile of immune effectors, and their biology in the breast mucosa, including both normal and hyperplastic breast mucosal tissue. Our goal in the present study is to understand the immune effector biology of breast mucosa (normal and cancer prone) and identify strategies which can enhance the infiltration of antigen-specific immune effectors targeting tumor cells in breast mucosa. As a part of this plan, we intend to use the transgenic mouse model of human breast cancer. We hypothesize that the mucosal immune system of the mammary gland can be modified to detect and eliminate potentially malignant cancer precursor cells. A thorough understanding of the immunity in breast mucosa will enable the design of appropriate vaccination strategies aimed at generating persistent mammary gland homing anti-cancer antigen-specific immune effectors. To test this general hypothesis we propose the following specific aims: **Specific Aim 1:** To determine the immune cell subsets in normal and hyperplastic breast in murine breast cancer models. **Specific Aim 2:** To determine whether immunization of mice with tumor antigen modifies the immune cell subsets and leads to persistent trafficking of tumor antigen-specific T and B cells into the mammary gland. **Specific Aim 3:** Determine an optimal oral vaccine approach able to minimize hyperplasia.

Progress report

Task 1: To determine the immune cell subsets in normal and hyperplastic breast in murine breast cancer models. The unique feature of gut mucosa is that its immune microenvironment is influenced by commensal bacteria which indicate that it is amenable to alteration in immune effectors which is the reason for the maintenance of gut homeostasis. Furthermore, gut associated T cells are not only involved in preventing colonization, but also homeostatic regulations of the epithelial layer. As part of the mucosal immune system, the mammary gland may have characteristic features similar to that of gut mucosa. Comparative gene expression and trafficking analysis in murine models has revealed several immune pathways related to mammary gland development during lactation and in the post lactation period.

Subtask 1a, Experiment 1: To determine immune cell subtypes in normal breast mucosa as compared to the small intestine. In this subtask, our goal was to determine if the mucosal effector immunity is similar in immune content to normal gut mucosa.

Single cell suspensions obtained from mammary pads, small bowel and spleen from different strains of mice (FVB, B6 and BALB/c mice) will be stained with antibodies specific for surface markers of different immune cells subsets and analyzed by flow cytometry using standard techniques. A minimum mouse sample size of 11 mice per group was used in these experiments and the results are the cumulative data of 3 independent experiments.

Finding #1: In B6 and Balb/C mice, the basic T cell composition of the breast mucosa is similar to that of the small intestine. In 6-8 week old mice, we analyzed the CD3$^{+}\alpha\beta$TCR^{+} T cells finding, as shown in **Figure 1**. In B6 and Balb/C mice, CD4 T cells constituted the bulk of the resident $\alpha\beta$TCR T cells in the mammary

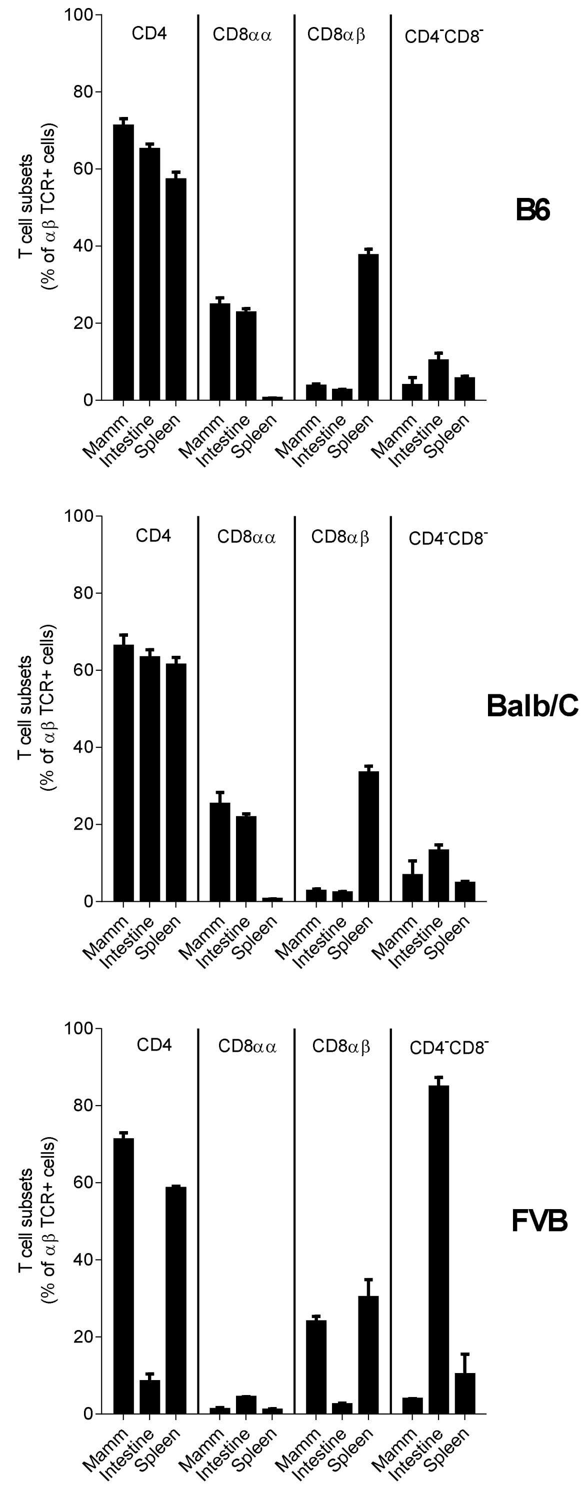

Figure 1: The mammary gland T cell composition is similar to other mucosal tissues. Shown are relative mean ($\pm$ s.e.m., n=12-18 mice) levels of CD4^{+}, CD8$\alpha\alpha^{+}$ CD8$\alpha\beta^{+}$, and CD4^{-}CD8^{-} T cells in the mammary gland (Mamm), small intestine, and spleen. Cells were gated on CD45, CD3 and the $\alpha\beta$ TCR.

gland, spleen, and small intestine. Like the intestine, in the mammary gland, the predominant CD8 T cell population was IELs (CD8αα$^+$) in B6 and Balb/C mice. In spleen, however, as expected, there were minor quantities of IELs and in that tissue the predominant CD8 T cell expressed the standard CD8αβ phenotype. Very few standard CD8 T cells were observed in these two mouse strains in the mammary gland. There were also in the mammary gland a small fraction of cells that expressed low to no CD4 or CD8. These double negative cells are in many respects similar to the CD8αα IELs according to prior studies. In contrast to the B6 and Balb/C, the FVB strain was significantly different with respect to the distribution of αβTCR T cells. While the predominant cell type was the CD4 T cell, the overwhelming majority of CD8 T cells appeared not to be CD8αα but rather CD8αβ. The distribution of T cells in the gut however, was markedly different with the vast majority being double negative (i.e., CD4$^-$CD8$^-$) with virtually no cells expressing either of the markers. These results indicate that there are some unique features of the FVB strain, which may be related to the fact that the FVB strain has a substantial fraction of the TCR gene locus deleted, a portion of the locus which is known to generate autoreactive TCRs.

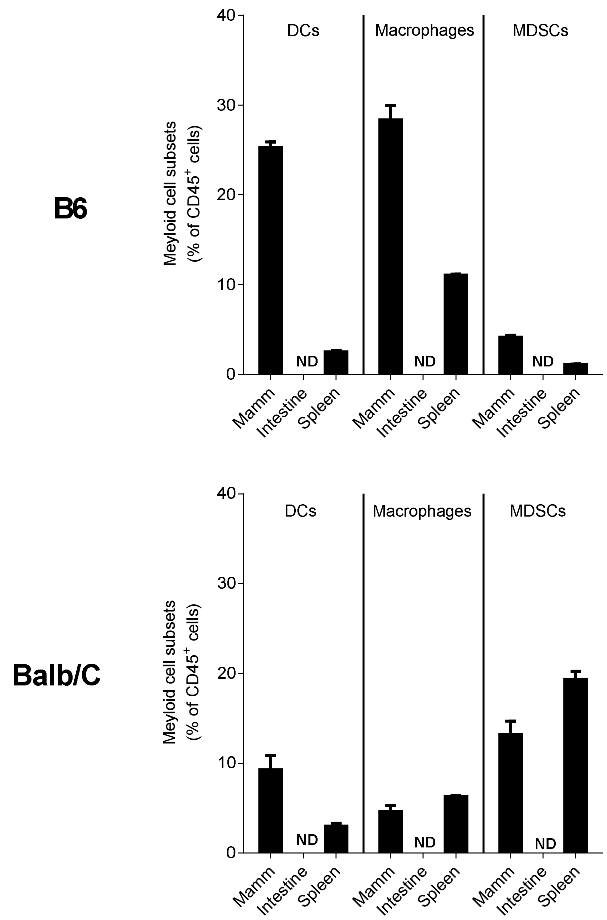

Figure 2: The mammary gland myeloid composition, relative to spleen, is elevated in B6 mice. Shown are relative mean (± s.e.m., n=12-18 mice) levels of DCs, Macrophages, and myeloid derived suppressor cells in the mammary gland (Mamm), and spleen. Cells were gated on CD45. ND=not done.

Finding #2: Myeloid cells infiltrate the mammary gland mucosa. We have also initiated comparisons of the levels and types of myeloid cells as shown in **Figure 2.** While this work is ongoing, we do see evidence of myeloid infiltration, specifically CD11c dendritic cells (DCs), macrophages, and myeloid-derived suppressor cells (MDSCs), into the mammary gland. This work is ongoing and at present the data is immature and we are unable to make conclusions.

Subtask #1a; Experiment 2: To determine the clonality of mammary gland infiltrating T cells. In this experiment, the oligoclonality of breast and gut epithelium associated intraepithelial lymphocytes (CD8αβ and CD8αα subsets) will be determined using sorted different T cell subsets and TCR Vβ repertoire CDR3 diversity repertoire analysis. We are currently in the process of setting up this experiment. With the transfer from VGTI to the Mayo Clinic, we have put this on hold as we will require the expertise of the genotyping core at the Mayo Clinic.

Subtask 1b: To determine the effect of estrous, pregnancy, age and breast feeding on the immune cell subsets of breast mucosa. Immunization to achieve optimal immune trafficking is likely to be impacted by those influences that are known to alter the gland biology. In this subtask the effect of different conditions (that can effect gland biology) in altering the immune cell subsets of breast mucosa is being determined.

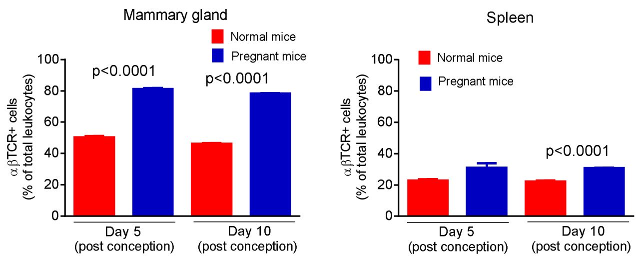

Figure 3: αβ TCR T cells are significantly increased during pregnancy. Shown are the mean (±s.e.m, n=6-12 mice) levels of αβ-TCR in the mammary gland and the spleen at 5 and 10 days of pregnancy (Blue) as compared with normal non-pregnant mice (Red).

Finding #1: Pregnancy is associated with a relative increase in the levels of αβ TCR T cells. Data collection for subtype 1b has moved along significantly, although we need to continue to collect the data. As shown in Figure 3, pregnancy is associated with relative increases in the levels of T cells. In those experiments, mammary glands and spleens were harvested from pregnant or age-matched control mice aged 6-8 weeks. The first day of pregnancy was based on the presence of a copulatory plug. Tissues were harvested on either day 5 or day 10. As shown, in the **Figure 3**, there is a dramatic, sustained increase in the relative numbers of αβ TCR T cells in the mammary gland but not spleen. When specific evaluating T cell subset, we found that both standard CD8αβ T cells and CD8αα IELs were relatively higher on day 5 but not day 10 in the mammary gland (**Figure 4**). CD8αβ T cells and CD8αα IELs in the spleen were slighted decreased in pregnant animals.

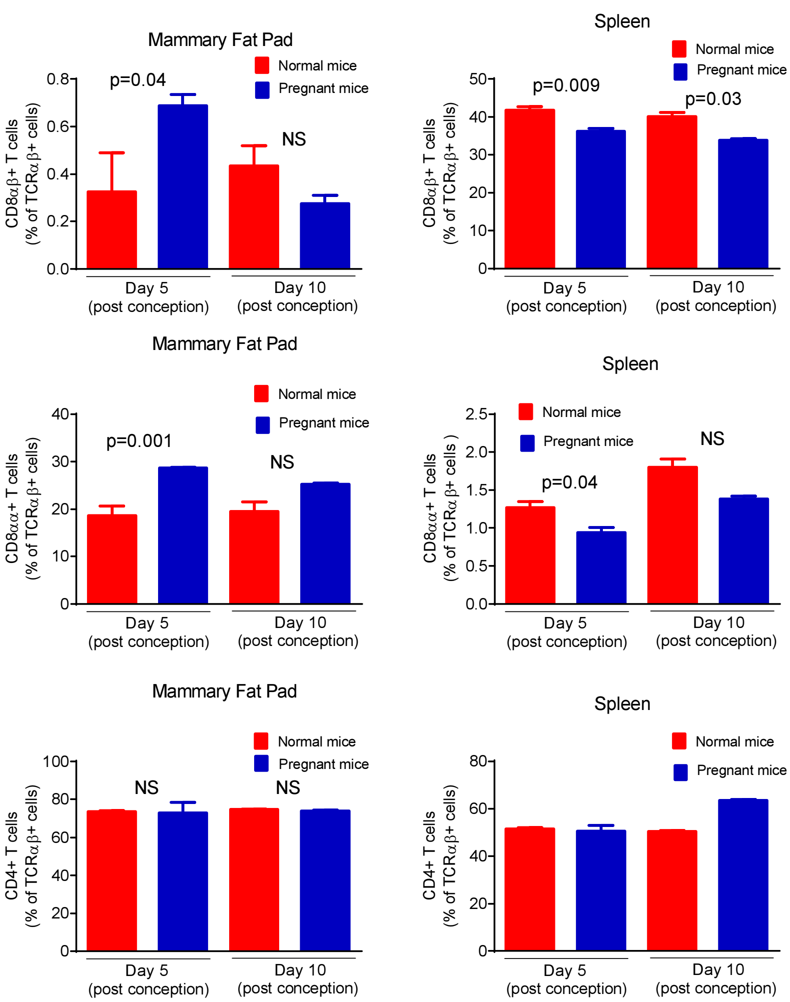

Figure 4: CD8 αα IELs and CD8 αβ T cells are significantly increased in the mammary gland during pregnancy. Shown are the mean (±s.e.m, n=6-12 mice) levels of αβ-TCR in the mammary gland 5 days into pregnancy (Blue) as compared with normal non-pregnant mice (Red).

Finding #2: There are no fluctuations in the mammary gland T cells during the different stages of estrus. The changes in the relative proportions of T cells in the mammary gland was apparently unaffected by the stage of

estrus (**Figure 5**). In this experiment, the stage of estrus was cytologically scored prior to harvesting tissues in the mouse. As shown in Figure 5, levels of CD4 and CD8αβ T cells and CD8αα IELs in the mammary gland were similar across all stages.

Finding #3: Lactation is associated with a substantial reduction in T cell infiltration into the mammary gland. We looked at whether there were any alterations in the gland as a result of lactation (**Figure 6**). In this experiment, we evaluated the distribution of T cells in the gland in mice that were lactating and nursing pups (At day 7-10 following initiation), mice that had just pups but which were removed prior to avoid lactation (at days 7-10 following removal of pups) and in normal age-matched control mice. As shown in **Figure 6A**, the relative levels of αβ TCR T cells sharply declined with lactation. In mice that had given birth but were not allowed to lactate (non-lactating), the total number of αβ-TCR T cells did not drop. Examining for CD8αα IELs, we found very sharp decreases (**Figure 6B)** in the relative amounts in both lactating and non-lactating CD8αα T cells accompanied by sharp increases in CD8αβ TCR T cells (**Figure 6C**). The proportion of CD4 T cells remained largely unchanged as a result of lactation (**Figure 6D).**

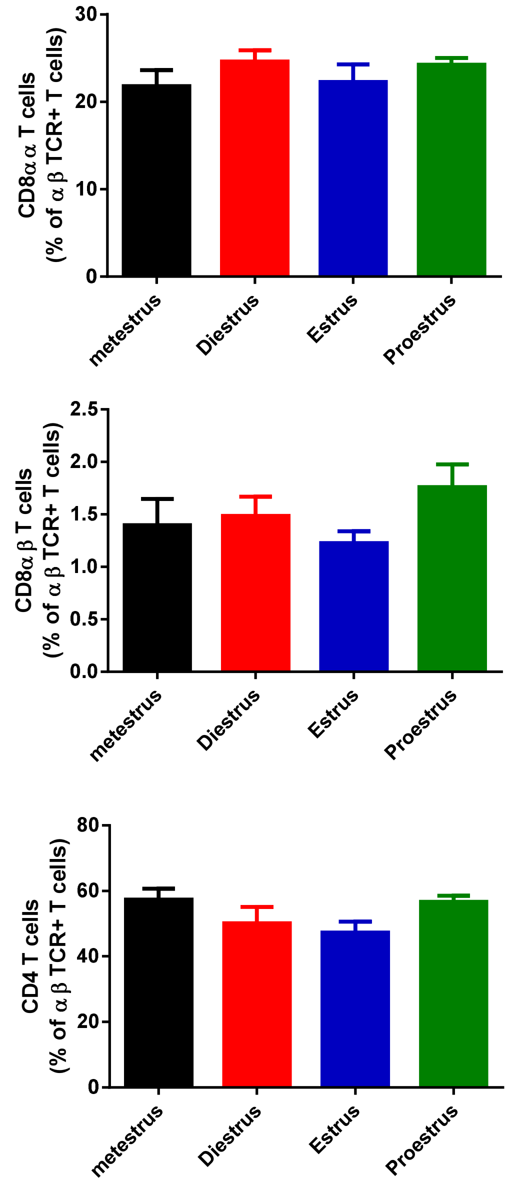

Figure 5: The proportions of CD8 αα IELs, CD8αβ, and CD4 T cells are not significantly changed in the mammary gland over the course of the estrus cycle. Shown are the mean (±s.e.m, n=10-18 mice) levels of αβ-TCR T cells in the mammary gland over the course of estrus.

Task 2: To determine whether immunization of mice with tumor antigen modifies the immune cell subsets and leads to persistent trafficking of tumor antigen-specific T and B cells into the mammary gland. In this specific task we are addressing the fundamental hypothesis as to whether the mammary gland mucosal immune microenvironment can be modified with vaccine to enrich for tumor-antigen-specific T and B cells. Trafficking of the two subsets i.e. conventional (CD4 or CD8αβ) αβ-TCR effector/memory T cells or CD8αα αβ(or γδ)-TCR T cells to mammary gland was also be examined.

Subtask 2b: To determine whether immunization leads to sustained lamina propria and intraepithelial immune memory. In the previous report, we showed that intra-glandular immunization leads to generation of immune responses. In this subtask, the goal is to determine whether tissue resident memory is established in the glandular tissue by performing a longitudinal analysis following intramammary vaccination. Thus, standard C57BL/6 mice were immunized between 6 and 8 weeks and tissues (mammary gland mucosa and lymph nodes and

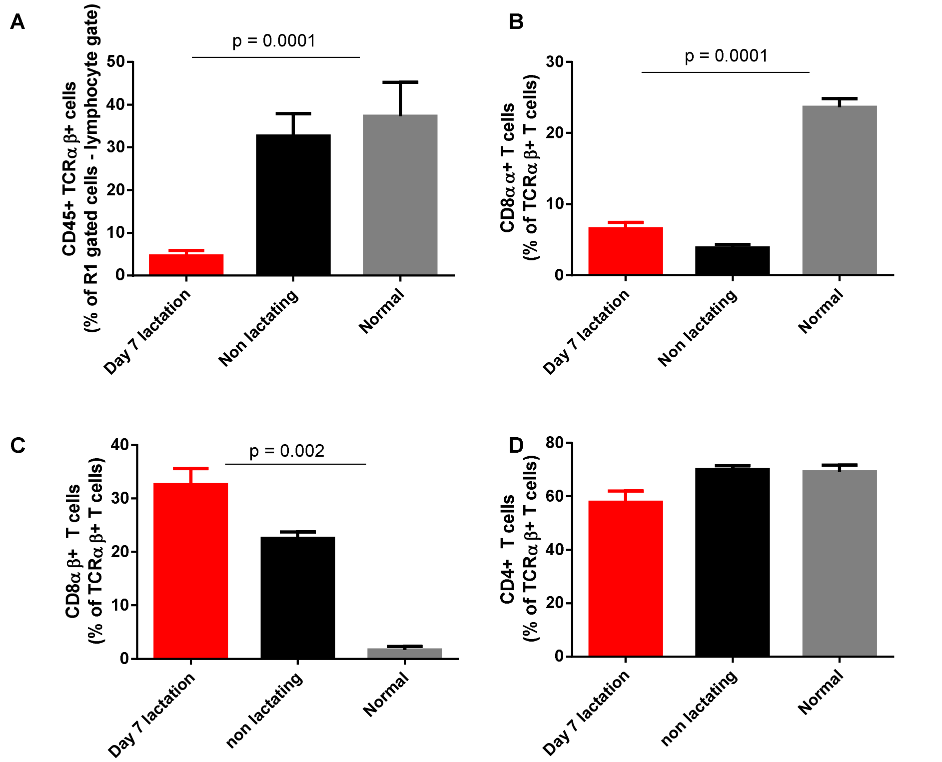

Figure 6: The proportions of CD8 αα IELs, CD8 αβ, and CD4 T cells are significantly changed in the mammary gland during lactation. Shown are the mean (±s.e.m, n=6-12 mice) levels of total CD45+ αβ-TCR T cells, CD8αα IELs, CD8 αβT cells and CD4 T cells in the mammary gland during lactation, in non-lactating moms and normal healthy mice.

spleen) were harvested at multiple time points following the final vaccine and single cell suspensions were analyzed. We estimate that a sample size of 11 mice per group will be used in this experiment and the experiment will be repeated at least 1-2 times.

Finding # 1: Vaccination directly into the mammary gland results in persistent immunity. For the current progress report we have evaluated up to 8 weeks following immunization. We immunized mice with either PBS (Control), TMEV virus (encoding no inserted T cell antigens), TMEV OT-I/OT-II which carry T cell epitopes, and free peptide OT-I/OT-II vaccine admixed with CFA/IFA. Three vaccinations were given over the course of 1 week. As shown in **Figure 7**, fairly strong T cell responses (both CD4 and CD8) were generated with the peptide vaccine but no good responses that could be detected 7 days after immunization could be detected when using TMEV encoding the antigens. Importantly, when mice were immunized and evaluated at 4 or 8 weeks following the last immunization, we were able to continue to detect responses although they had significantly waned indicating the need for booster immunizations. The reason for the strong immune responses was due to a large influx of large number of αβ–TCR T cells, specifically in the peptide vaccine group, as shown in **Figure 8**. Further analysis of the T cells reveals that the vaccine induced influx is due to infiltration of αβ-TCR, CD8αβ T cells, whereas there was no increase in either CD8αα or CD4 T cells.

The Key Research Accomplishments

- Demonstrated that the mammary gland distribution of T cells (in Balb/c and B6) mice is similar to that observed in the gut rather than the spleen.
- That pregnancy is associated with an influx of αβ–TCR T cells and that the increase in due to both CD8αα and CD8αβ T cells, but not CD4 T cells.
- The relative frequencies of T cells do not change with the stage of estrus.
- Lactation is associated with a decrease in the numbers αβ-TCR T cells with sharp decreases in the proportion of CD8αα T cells.

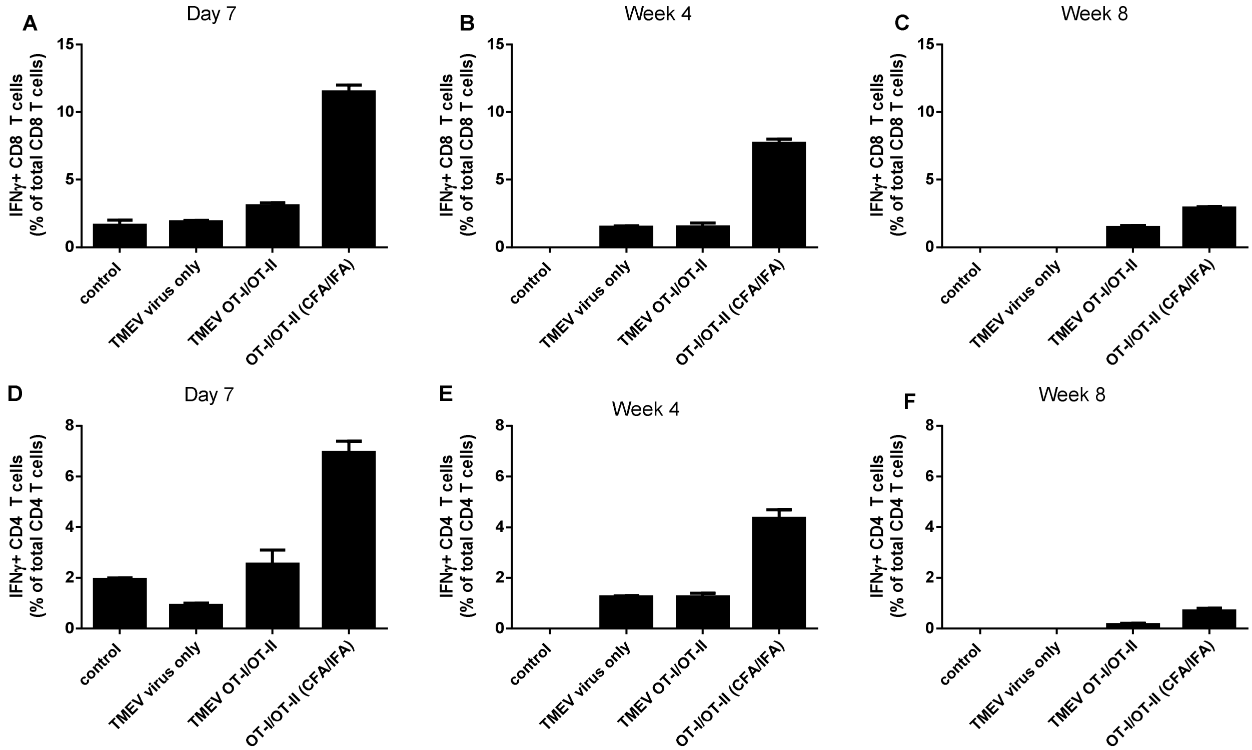

Figure 7: Direct intramammary peptide vaccination leads to sustained antigen-specific immunity in the mammary gland. Shown are the mean (±s.e.m., n=6 mice) levels of antigens specific T cells in the mammary gland that recognize OT-1 (Panels A-C) and OT-11 (Panels D-F) epitopes at Day 7 (Panels A and D), week 4 (Panels B and E), and Week 8 (Panels C and F). Antigen-specific T cells were assess using flow cytometric staining of intracellular cytokines.

- Vaccination directly into the mammary gland with peptide antigen leads to sustained immune responses lasting for several weeks.

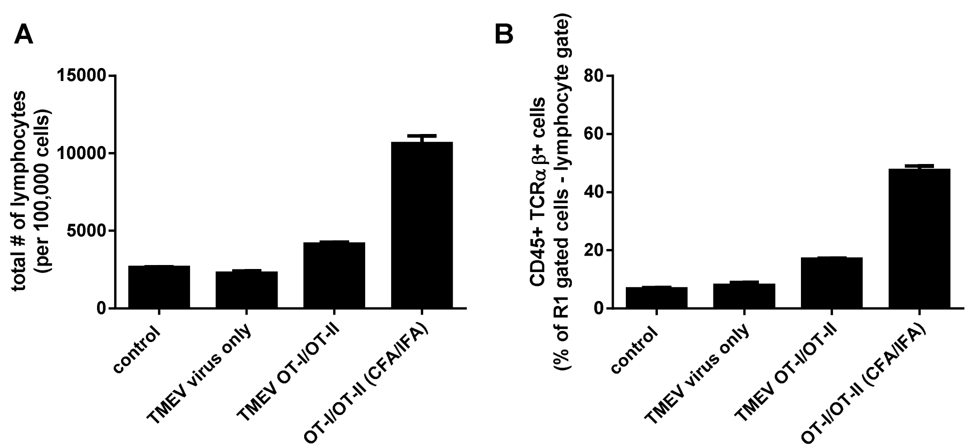

Figure 8: Direct intramammary peptide vaccination leads to infiltration of $\alpha\beta$-TCR T cells in the mammary gland. Panel A: Shown are the mean (±s.e.m., n=6 mice) absolute levels of lymphocytes in the mammary gland following immunization as described above for Figure 7. **Panel B** shows the percentage of the cells in Panel A that are $\alpha\beta$-TCR expressing T cells.

Reportable outcomes:

All new findings in this report are novel and reportable. We will combine with data collected in the next period and report in a major scientific journal.

Presentations at meetings:

None

Publications in scientific journals:

None

Conclusions

Our goal is to develop preventative breast cancer vaccine. Given the fact that breast cancer originates from mucosal tissue and it is well known that mucosal tissue associated with gut is endowed with intact immune system, we proposed, in this grant, to study the immune effectors associated with breast mucosa. Based on this information our goal was to develop strategies to educate breast mucosa using different vaccination approaches. In order to stimulate immunity specifically in the breast, we chose in this funding period to explore the distribution of T cells in the breast as a function of reproduction and lactation, which could influence response to vaccination. This is enabling the identification of the most appropriate times to immunize. We also demonstrate for the first time that immunization into the breast elicits immunity that persists for many weeks following vaccination.

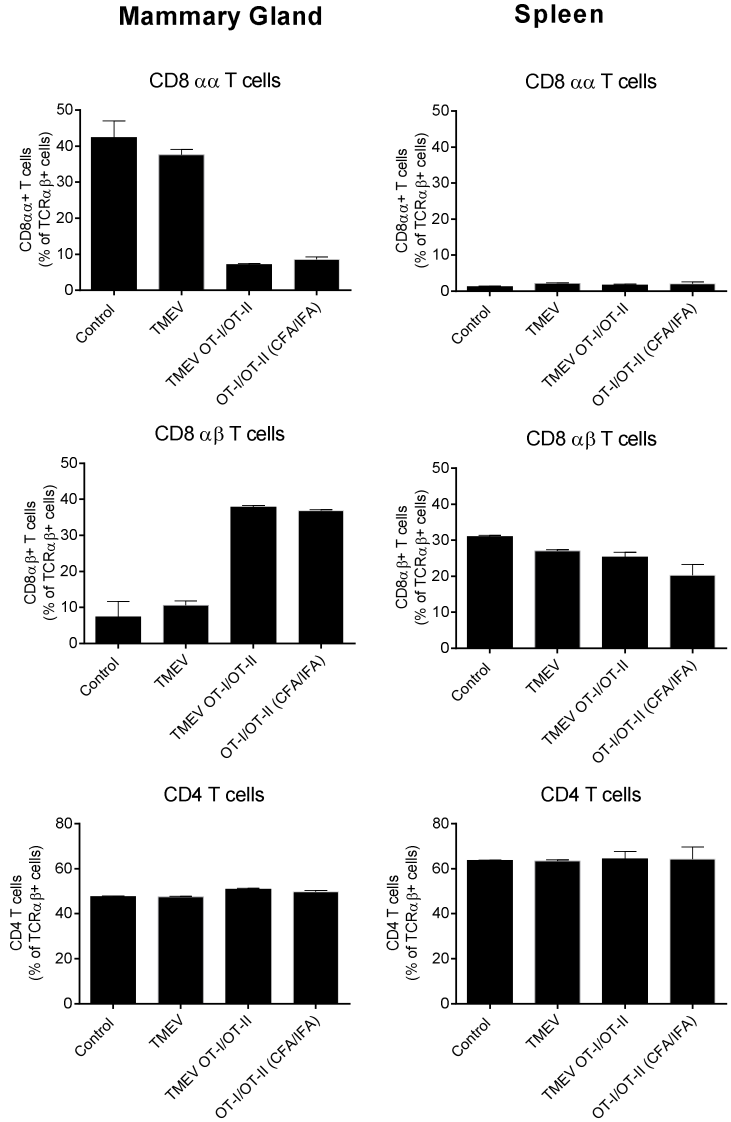

Figure 9: Direct intramammary peptide vaccination leads to infiltration of αβ-TCR, CD8 αβ T cells into in the mammary gland. Shown are the relative mean (±s.e.m., n=6 mice) levels of CD8αα, CD8αβ and CD4 T cells in the mammary gland and spleen at 1 week following immunization as described for Figure 7.

Principal Investigator Statement:

Overall, the progress of this project has been excellent to date. Our staff and I are excited and optimistic about the refinements that we have made to the project. The aims that we have chosen are novel, cutting edge, and challenging to us. The work encompassed in the present report was done exclusively at the Vaccine and Gene therapy Institute of Florida and adds to the data accumulated from the initial year of the award (i.e., data that was collected at Mayo Rochester). Over the next funding period (at Mayo Clinic Jacksonville) we will eagerly integrate the results obtained in the two previous periods and finish the proposed experiments as some remain uncompleted with respect to the final animal numbers that we need to achieve to make strong statistically supported statement. We will then finalize the study with testing of oral immunization routes to see if that feasibly generate immunity that can traffic to the mammary gland. The results are expected to result in a publication and enable access to future funding testing the novel idea that we can prevent breast cancer by immunizing into the gut or directly into the mammary gland.

References

None

Appendices

None